

L1, a novel target of β -catenin signaling, transforms cells and is expressed at the invasive front of colon cancers

Nancy Gavert,¹ Maralice Conacci-Sorrell,¹ Daniela Gast,² Annette Schneider,² Peter Altevogt,² Thomas Brabletz,³ and Avri Ben-Ze'ev¹

¹Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, 76100, Israel

²Tumor Immunology Programme, D010 German Cancer Research Center, D-69120, Heidelberg, Germany

³Department of Pathology, University of Erlangen-Nürnberg, 91054 Erlangen, Germany

A aberrant β -catenin-TCF target gene activation plays a key role in colorectal cancer, both in the initiation stage and during invasion and metastasis. We identified the neuronal cell adhesion molecule L1, as a target gene of β -catenin-TCF signaling in colorectal cancer cells. L1 expression was high in sparse cultures and coregulated with ADAM10, a metalloprotease involved in cleaving and shedding L1's extracellular domain.

L1 expression conferred increased cell motility, growth in low serum, transformation and tumorigenesis, whereas its suppression in colon cancer cells decreased motility. L1 was exclusively localized in the invasive front of human colorectal tumors together with ADAM10. The transmembrane localization and shedding of L1 by metalloproteases could be useful for detection and as target for colon cancer therapy.

Introduction

The development of human cancer is considered a multistage process involving genetic changes endowing the tumor cells first, with proliferative advantage and, at later stages, with the capacity to breakdown cell–cell adhesions, and with motile capacity, enabling the cancer cells to invade neighboring tissues. In colorectal cancer, an early event is the aberrant activation of β -catenin-TCF signaling which results from mutations in β -catenin, or its degradation machinery, thereby leading to the accumulation of β -catenin in the nucleus where it forms transcriptionally active complexes with LEF/TCF factors (Bienz and Clevers 2000; Polakis, 2000; Conacci-Sorrell et al., 2002a). Hyperactivation of growth promoting target genes of β -catenin-TCF signaling, including *Cyclin D1* (Shtutman et al., 1999; Tetsu and McCormick, 1999) and *c-myc* (He et al., 1998) may promote the onset of oncogenesis.

Inappropriate activation of β -catenin signaling also contributes to later stages of tumorigenesis by inducing genes that confer invasive and metastatic capacities. These include metalloproteases (Brabletz et al., 1999; Crawford et al., 1999; Takahashi et al., 2002; Hlubek et al., 2004), ECM components (Gradl et al., 1999; Hlubek et al., 2001) and cell adhesion

receptors such as CD44 (Wielenga et al., 1999), Nr-CAM (Conacci-Sorrell et al., 2002b), and uPAR (Mann et al., 1999). In addition to its role as cotranscriptional activator, β -catenin is a major component of adherens junctions linking cadherin family transmembrane receptors to the actin cytoskeleton (Ben-Ze'ev and Geiger, 1998). By playing a dual role in cell adhesion and transcriptional regulation, β -catenin can integrate changes in these two key cellular processes that are disrupted in cancer cells.

Recent studies of human colorectal cancer tissue support this view by demonstrating reversible changes in E-cadherin and β -catenin localization during metastasis. Cells at the central tumor mass in the primary tumor display polarized epithelial organization, and form tubular structures with junctional localization of β -catenin and E-cadherin, whereas cells at the invasive front are characterized by loss of cell-surface E-cadherin and nuclear localization of β -catenin (Brabletz et al., 2001). Interestingly, in lymph node metastases, these two phenotypes of cellular organization seen in the primary tumor are regained, suggesting plasticity in colon cancer cellular morphogenesis during metastasis (Barker and Clevers, 2001; Brabletz et al., 2001). Using a model system of colon cancer cells grown at varying densities in vitro, we could mimic these two different forms of cellular organization and identified signaling pathways linking the negative regulation of E-cadherin expression

Correspondence to Avri Ben-Ze'ev: avri.ben-zeev@weizmann.ac.il

Abbreviations used in this paper: Δ NLEF, dominant negative LEF-1; Δ NTCF, dominant negative TCF.

with nuclear signaling by β -catenin (Conacci-Sorrell et al., 2003). Sparse cultures of colon cancer cells express only small amounts of E-cadherin and high levels of nuclear β -catenin, reminiscent of colon cancer cells at the invasive tumor front, whereas dense cultures have well-developed E-cadherin and β -catenin-containing adherens junctions with little nuclear β -catenin resembling the central, more differentiated part of colorectal tumors (Brabletz et al., 2001; Conacci-Sorrell et al., 2003).

In the present study, we used this cell culture system to characterize a novel target gene of β -catenin signaling involved in human colon cancer invasion, and determined the localization of its gene product in colon cancer tissue. We identified L1, a transmembrane cell adhesion molecule, normally expressed in nerve cells, as a target of β -catenin-TCF signaling, and showed that its expression confers cell motility, invasion and tumorigenesis in fibroblasts and colon cancer cells. In colorectal cancer tissue, L1 was exclusively localized at the invasive front of the tumor tissue that expresses nuclear β -catenin, together with the metalloprotease ADAM10 that is involved in the cleavage and shedding of the L1 extracellular domain.

Results

Cell density-dependent expression of L1 and ADAM10 in colon cancer cells

We used the density-dependent phenotypic conversion displayed by two human colon cancer cells lines, SW480 and HCT116, that mimic the changes in E-cadherin and β -catenin localization, and in E-cadherin expression, displayed by colon cancer cells in the differentiated part and the invasive front of colorectal tumors (Brabletz et al., 2001; Conacci-Sorrell et al., 2003). SW480 cells have mutant APC, and wt β -catenin accumulates in the nuclei of these cells in sparse cell cultures (Fig. 1 C). In HCT116 cells, a stabilizing mutation in the NH₂ terminus of β -catenin (on Ser45) is responsible for its accumulation and increased nuclear signaling (Morin et al., 1997). When looking for novel β -catenin target genes that correlate with the invasive phenotype of human colon cancer cells, we reasoned that L1 might be a good candidate, as it is among genes whose expression decreased in a DNA microarray analysis of SW480 cells transduced with wt APC (Lin et al., 2001). In addition, we recently identified another member of the L1 family, Nr-CAM, as a target gene of β -catenin, and showed that its expression is induced in human melanoma and colon carcinoma (Conacci-Sorrell et al., 2002b).

We compared the expression of L1 in sparse and dense cell cultures of both SW480 and HCT116 cells and found that sparse cells express higher levels of L1 than dense cells (Fig. 1 A), whereas E-cadherin expression behaved in the opposite manner. The higher level of L1 in sparse cultures resulted from increased L1 RNA in these cells (Fig. 1 B). Sparse SW480 cells displayed strong cytoplasmic L1 distribution, often in large vesicles (Fig. 1 C, arrows), whereas in dense cultures, a weaker staining of L1 was found in the membrane at cell-cell contact sites, consistent with the capacity of L1 to

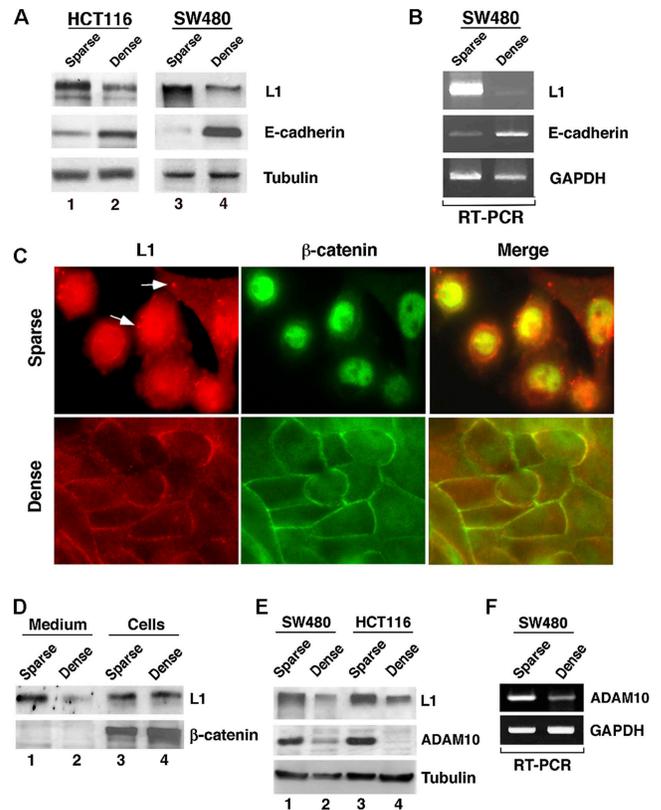


Figure 1. Coregulation of L1 and ADAM10 expression by cell density in human colon cancer cells. (A) Expression of L1 in sparse and dense cultures of HCT116 and SW480 cells was compared with E-cadherin. Tubulin served as loading control. (B) RNA levels were determined by RT-PCR. (C) The localization of L1 and β -catenin in sparse and dense cultures was compared by double immunostaining for L1 and β -catenin (arrows point to L1 in vesicles). (D) The levels of L1 in culture medium of sparse and dense cultures were compared by loading equivalent volumes of medium corresponding to cells expressing equal amounts of cell-associated L1 (lanes 3 and 4). More medium and cell lysates from dense cultures were required to obtain an equal amount of cell-associated L1 (see the level of β -catenin). (E) L1 and ADAM10 levels were compared in sparse and dense cultures of SW480 and HCT116 cells. (F) ADAM10 RNA levels in sparse and dense cultured of SW480 cells were determined by RT-PCR with GAPDH RNA serving as loading control.

mediate homophilic cell-cell interactions. However, this localization of L1 in dense cells did not coincide significantly with adherens junctions visualized by staining for β -catenin (Fig. 1 C, dense, merge). The presence of L1 in vesicles and its reported shedding from the surface of some tumor cells mediated by the metalloprotease ADAM10 (Mechtersheimer et al., 2001), prompted us to examine whether L1 can be found in the culture medium of sparse and dense colon cancer cells. When the medium was analyzed from cells expressing equivalent amounts of cell-associated L1 (Fig. 1 D, lanes 3 and 4), we found much more L1 shedded into the medium of sparse than dense cell cultures (Fig. 1 D, lanes 1 and 2). Moreover, in both SW480 and HCT116 cells more ADAM10 was found in sparse cell cultures (Fig. 1 E), and correlated with the higher levels of ADAM10 RNA in such cells (Fig. 1 F). These results indicate that L1 might be a novel target gene of β -catenin signaling, coexpressed with ADAM10 in sparse

cell cultures that mimic the invasive phenotype of human colon carcinoma.

The L1 promoter is activated by β -catenin-TCF signaling

We wished to determine whether the *L1* gene is a target for activation by β -catenin-TCF signaling. When an *L1* promoter reporter plasmid containing 2.9-kb upstream of the transcription initiation site (Fig. 2 C; Kallunki et al., 1997; Meech et al., 1999) was cotransfected with a point mutant (S33Y) stabilized β -catenin into 293 cells, it was activated sixfold (Fig. 2 A), similar to the *Cyclin D1* promoter reporter that served as control for a previously characterized β -catenin target gene (Shtutman et al., 1999). Cotransfection of the cytoplasmic tail of cadherin, that binds to and sequesters β -catenin from binding to LEF/TCF factors (Sadot et al., 1998), inhibited *L1* transactivation. The involvement of LEF/TCF factors in *L1* transactivation is suggested by the inhibition of *L1* promoter activation using a dominant negative LEF-1 (Δ NLEF), whereas a dominant positive LEF-1 construct containing the DNA-binding domain of LEF-1 linked to the transactivation domain of viral VP16, activated the *L1* promoter (Fig. 2 A, LEF1/VP16). Transactivation of the *L1* promoter by endogenous β -catenin-TCF complexes in SW480 and HCT116 colon cancer cells was inhibited by cotransfection of components involved in β -catenin degradation (APC and Axin), and also by the cytoplasmic cadherin tail (Fig. 2 B). Both, the *Cyclin D1* promoter and the synthetic TOPFLASH reporter plasmid, containing several LEF/TCF-binding sites, displayed similar responses in these experiments (Fig. 2 A and B). We identified 4 putative TCF-binding sites in the 2.9-kb of the *L1* promoter (Fig. 2 C). In DNA electrophoretic mobility shift analyses, all 4 putative TCF-binding sites bound to protein complexes containing β -catenin-TCF derived from nuclear extracts of SW480 cells (the data for radiolabeled site no. 4 are shown in Fig. 2 D). When radiolabeled site no. 4 was incubated with nuclear extracts from SW480 cells and with antibody to β -catenin, a supershift band was detected (Fig. 2 D, lane 8), indicating the presence of β -catenin in this DNA-protein complex. This binding was inhibited by incubation with increasing concentrations of the TCF-binding site of the *Cyclin D1* promoter (Fig. 2 D, compare lanes 3–5 with lane 2).

Next, we examined whether endogenous β -catenin-TCF signaling activates the endogenous *L1* gene of colon cancer cells, using HCT116 cells expressing doxycycline inducible siRNA to β -catenin and dominant negative TCF4 (Δ NTCF; van de Wetering et al., 2003). The efficiency of this strategy in suppressing β -catenin-TCF signaling was demonstrated by reduced TOPFLASH activity 2 d after doxycycline-mediated induction of Δ NTCF or β -catenin siRNA (Fig. 2 E). The siRNA to β -catenin also reduced the level of β -catenin (Fig. 2 F, compare lanes 7 and 8 with lanes 5 and 6). Analysis of *L1* expression in such HCT116 cells, where β -catenin levels were suppressed, revealed a major decrease in *L1* (Fig. 2 F, lanes 7 and 8). Similarly, when TCF-mediated signaling was inhibited by inducible Δ NTCF4, *L1* expression was also diminished (Fig. 2 F,

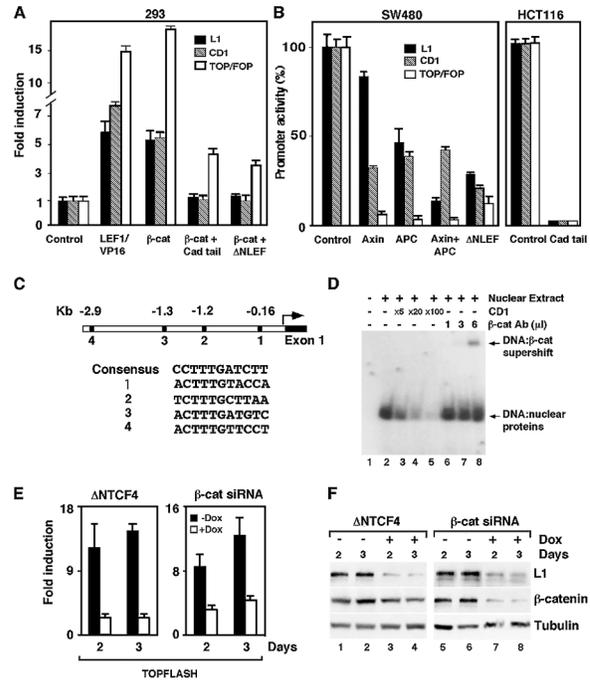


Figure 2. The *L1* gene promoter is activated by β -catenin-TCF signaling in colon cancer cells. (A) The *L1*, *Cyclin D1*, and TOPFLASH promoter reporter plasmids were transfected into 293 cells together with β -galactosidase that served to normalize for transfection efficiency. Cells were cotransfected with either a stabilized, activated β -catenin S33Y mutant (β -cat), with mutant β -catenin and the cytoplasmic tail of cadherin (Cad tail), or with Δ NLEF lacking the DNA-binding domain (Δ NLEF). Cells were also cotransfected with these gene reporters and a dominant positive LEF1/VP16 construct. Fold activation for TOPFLASH was determined after dividing promoter activity by the values obtained with the mutant FOPFLASH construct. For the other gene promoters, the values were divided by those obtained with empty vector. (B) Activation of *L1* and the other gene promoters described in A by endogenous β -catenin-TCF complexes in SW480 and HCT116 colon cancer cells. Cells were transfected with the promoter reporter plasmids in the presence or absence of cDNAs encoding for components that enhance β -catenin degradation (APC, Axin), Δ NLEF, and the cytoplasmic tail of cadherin (Cad tail) that sequesters β -catenin from binding to TCF. (C) Schematic representation of putative TCF-binding sites in the *L1* promoter. (D) DNA mobility shift assays with nuclear extracts from SW480 cells incubated with 32 P-radiolabeled TCF-binding site no. 4 of the *L1* promoter and increasing concentrations of competing non-radioactive *Cyclin D1* promoter TCF-binding sequences (CD1) (lanes 3–5), or increasing concentrations of anti- β -catenin antibody (lanes 6–8). (E) Activity of transfected TOPFLASH in HCT116 cells after doxycycline (Dox)-mediated induction of Δ NTCF4 and β -catenin siRNA. Values were determined 2 and 3 d after induction. (F) Levels of *L1* and β -catenin in HCT116 cells expressing inducible Δ NTCF4 and β -catenin siRNA. Error bars represent one SD.

compare lanes 3 and 4 with lanes 1 and 2). Together, these data argue that β -catenin-TCF signaling can induce the *L1* gene of human colon cancer cells.

Expression of L1 in fibroblasts confers enhanced motility, growth in low serum, transformation, and tumorigenicity in nude mice

To understand the possible role(s) of *L1* in development of the tumorigenic phenotype, we considered the known functions of *L1* in nerve cells, where it is involved in neuronal migration, axonal growth, and guidance (Kamiguchi et al.,

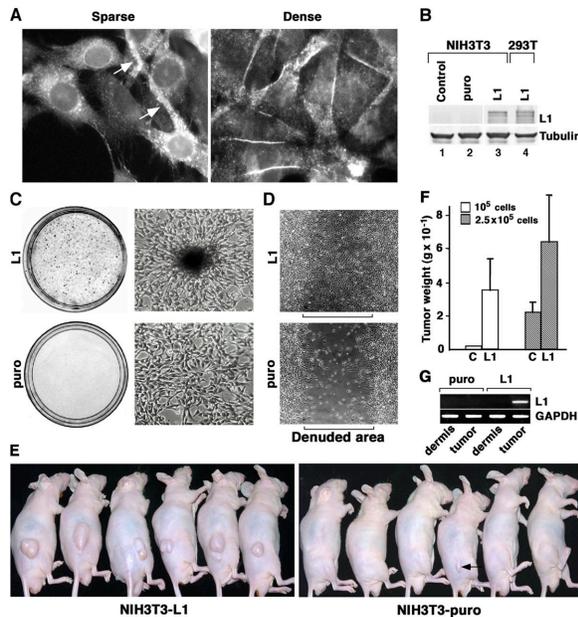


Figure 3. Expression of L1 in NIH3T3 cells confers enhanced motility, transformation and tumorigenesis in nude mice. (A) NIH3T3 cells were infected with a retrovirus construct coding for L1 and the puro^r gene, or puro^r alone, and puro^r cells were selected. The organization of L1 in these cells was determined by staining with antibody to L1 in sparse and dense cell cultures. (B) Expression of L1 in NIH3T3 cells was compared (by Western blotting) to that of 293T cells transiently transfected with L1 cDNA. (C) The growth of L1 expressing cells that formed foci is compared with puro^r controls at low and high magnification under a microscope. (D) The motile capacity of L1 expressing NIH3T3 cells expressing puro^r alone, or together with L1, was compared in closing an artificial wound introduced into a confluent monolayer. The picture was taken 16 h after “wounding” the monolayer. (E) The ability of NIH3T3-puro^r cells and cells expressing L1 (NIH3T3-L1) to form tumors was determined by injecting (subcutaneously) groups of six mice with 10⁵ and 2.5 × 10⁵ cells, and (F) by measuring tumor weight after 2 wk. Error bars represent one SD. (G) Expression of L1 RNA in the tumor tissue and dermis was determined by RT-PCR.

1998). To examine if L1 expression can affect the motility of other cells, we infected NIH3T3 cells with a retrovirus expressing L1, and selected cells resistant to a cotransfected puromycin-resistance gene (Fig. 3 B, puro). As shown in Fig. 3 A, NIH3T3 cells transduced with L1 displayed L1 in the cytoplasm (when grown at low density), mostly in vesicles, and in long cellular processes (Fig. 3 A, arrows). This organization was reminiscent of the vesicles seen in SW480 cells (Fig. 1 C). At higher cell density, L1 localized at cell–cell contacts (Fig. 3 A, dense), as seen in dense cultures of colon cancer cells (Fig. 1 C). Most significantly, L1 expressing NIH3T3 cells formed foci when grown on plastic, whereas puro^r cells did not (Fig. 3 C). We examined whether L1 expression influences the motility of NIH3T3 cells by introducing a gap in a confluent monolayer of cells, and followed the rate at which control and L1 expressing cells filled the gap. L1 expressing NIH3T3 cells moved into the denuded area much faster than puro^r control cells (Fig. 3 D). Because NIH3T3 cells expressing L1 formed foci (Fig. 3 C), indicating that L1 can confer cell transformation, we examined if these cells are tumorigenic in nude mice. We found that all six mice injected with L1 expressing cells formed tumors, whereas only one mouse devel-

oped a small tumor when injected with the puro^r control cells (Fig. 3 E, arrow). When higher numbers of cells were injected into mice, puro^r-expressing cells also formed tumors (Fig. 3 F), a property inherent to transduction with retroviral constructs. However, tumors formed by puro^r cells were at least three times smaller than those formed by L1-expressing cells (Fig. 3 F). Analysis of RNA from tumor tissue revealed that L1 is expressed in the tumors obtained with NIH3T3 cells expressing L1 (Fig. 3 G).

We examined the growth properties of NIH3T3 cells expressing L1 and found that in 10% of serum there were no differences between the growth rates of these cells and either control NIH3T3 cells, or puro^r cells (Fig. 4 A). In 0.5% of serum however, L1 expressing cells continued to grow and formed foci (Fig. 4 C), whereas control NIH3T3 and puro^r cells died after a few days (Fig. 4 B). We wished to determine which signaling pathways enabled L1 expressing NIH3T3 cells to grow in low serum. Cells were incubated in 0.5% of serum for 24 h and the levels of total ERK and activated (phosphorylated) ERK were determined. L1 expressing cells displayed constitutively activated ERK, whereas puro^r cells had no detectable P-ERK (Fig. 4 D, compare lane 1 with lane 2). When these cells were grown in 0.5% of serum and incubated with 10% of serum to induce ERK activation, both puro^r and L1 expressing cells maintained the ability to induce P-ERK upon serum stimulation (Fig. 4 D, lanes 3–8). This transient induction of ERK activation declined to undetectable levels by 24 h in puro^r cells, whereas L1 expressing cells continued to display sustained levels of P-ERK (Fig. 4 D, lanes 9 and 10). These results on ERK activation by L1 are in agreement with recent studies by Silletti et al. (2004).

In a previous study, expression of L1 was detected among genes suggested to be required for the growth of a variety of tumor, but not normal cells (Primiano et al., 2003). Hence, we examined if L1 is necessary for SW480 and HCT116 cell proliferation by incubating these cells with an antibody recognizing the extracellular domain of L1. As control, cells were incubated with denatured antibody (by boiling to destroy its activity). The L1 antibody blocked very effectively the proliferation of both SW480 and HCT116 cells (Fig. 4, E and F), suggesting that L1 expression is essential for colorectal cancer cell growth.

Overexpression of L1, or its suppression, affect cell motility, invasion, and tumorigenesis of colon cancer cells

To examine if L1 plays a role in determining the motile, invasive, and tumorigenic capacity of human colon cancer cells, we expressed L1 in LS174T human colon cancer cells that do not normally express L1 (Fig. 5 A), and found that L1 is mainly localized at cell–cell contact sites of such dense cell cultures (Fig. 5 B). Two independently isolated LS174T clones expressing L1 displayed an increased capacity to close an artificial wound introduced in a monolayer compared with control, neo^r cells (Fig. 5 C). When LS174T-neo^r cells were injected into one side of six nude mice, the L1 expressing cells

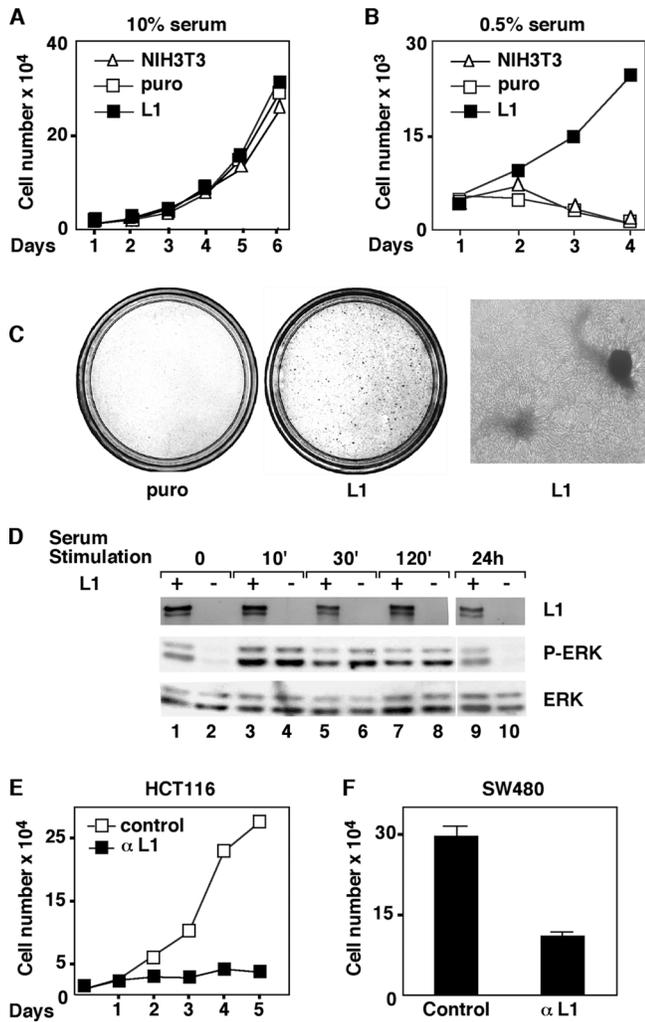


Figure 4. L1 can regulate cell proliferation, survival and MAPK activation. The proliferation of control NIH3T3 cells, puro^r expressing cells and cells expressing L1 was compared in the presence of 10% (A) and 0.5% (B) serum. (C) NIH3T3 cells expressing L1 formed foci when grown in 0.5% of serum on plastic, but puro^r cells did not. (D) NIH3T3 cells expressing L1 maintained a constitutive level of activated ERK (P-ERK) (lanes 1 and 9), whereas puro^r cells did not (lanes 2 and 10). Activation of ERK by serum stimulation, in cells grown for 2 d in 0.5% of serum, was similar in puro^r and L1 expressing cells (lanes 3–8). An antibody to the extracellular domain of L1 inhibited HCT116 (E) and SW480 (F) cell growth. Denatured L1 antibody (by boiling) served as control. Error bars represent one SD.

injected on the other side formed much larger tumors (Fig. 5 D). This was observed with two distinct LS174-L1 cell clones (Fig. 5 E). Using another colon cancer cell line, SW707, that also lacks L1 (Fig. 5 F), we compared the haptotactic motility (toward fibronectin) in transwell filter assays before, and after their stable transfection with L1. We found that such cells display enhanced haptotactic motility compared with empty vector transfected cells (Fig. 5 G). Moreover, L1-transfected SW707 cells were more invasive through filters coated with matrigel than control SW707 cells (Fig. 5 H), and when injected into mice formed larger tumors (Fig. 5 I), indicating that L1 can confer enhanced ECM invasion and tumor growth also in these colon cancer cells. In contrast, suppression of endogenous L1 levels in HCT116 cells by L1 siRNA oligonucle-

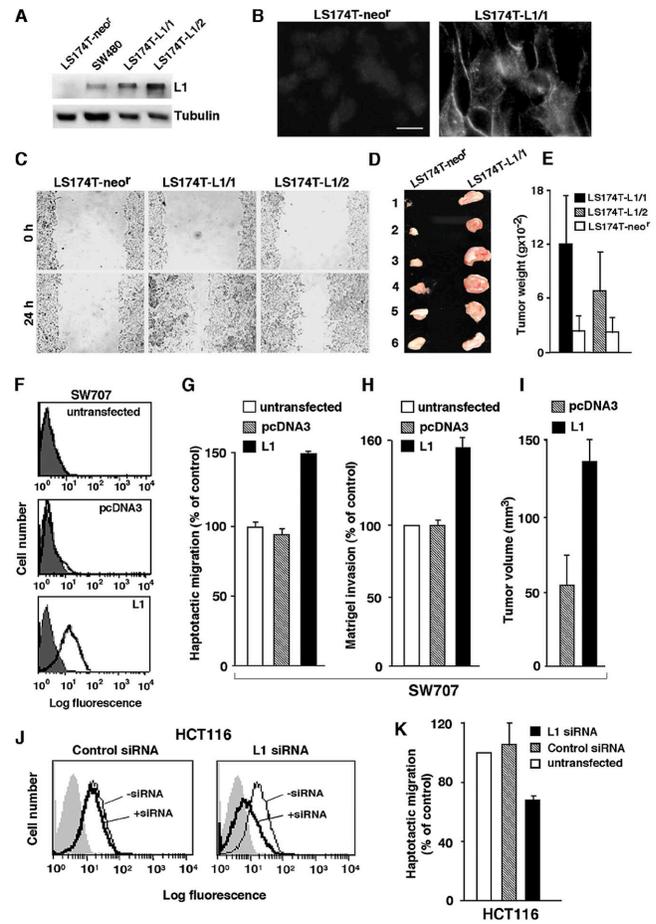


Figure 5. L1 expression in colon cancer cells enhances their migration, ECM invasion and tumorigenesis, whereas its suppression inhibits cell motility. (A) LS174T colon cancer cells lacking L1 were stably transfected with neo^r alone (LS174T-neo^r), or together with L1 and individual L1-expressing clones (LS174T-L1/1; LS174T-L1/2) and neo^r clones were identified by Western blotting and compared with endogenous L1 levels expressed by SW480 cells. (B) Transfected L1 was localized at cell-cell contacts in LS174-L1 cells. Bar, 10 μ m. (C) The ability of LS174-L1 cell clones to close an artificial wound was enhanced and (D and E) these cells formed larger tumors when injected into nude mice compared with neo^r cells. (F) SW707 colon cancer cells lacking L1, when stably transfected with L1 displayed enhanced haptotactic motility toward fibronectin (transwell analysis; G), increased invasion through matrigel (H), and increased capacity to form tumors in nude mice (I). HCT116 colon cancer cells in which endogenous L1 levels were suppressed by siRNA (J) had reduced haptotactic motility compared with control (K). Error bars represent one SD.

otides (Fig. 5 J) led to decreased haptotactic motility (Fig. 5 K), whereas further elevation of L1 in HCT116 cells, by adenovirus-mediated infection, increased their motility (not depicted). Together, these results imply that L1 expression in human colon cancer cells plays an important role in their motile, invasive and tumorigenic capacities.

Expression of L1 and ADAM10 at the invasive front of human colorectal tumor tissue

We wished to detect and localize the expression of L1 in human colorectal cancer tissue. For this, we performed histochemical staining with antibodies to L1 and β -catenin in serial

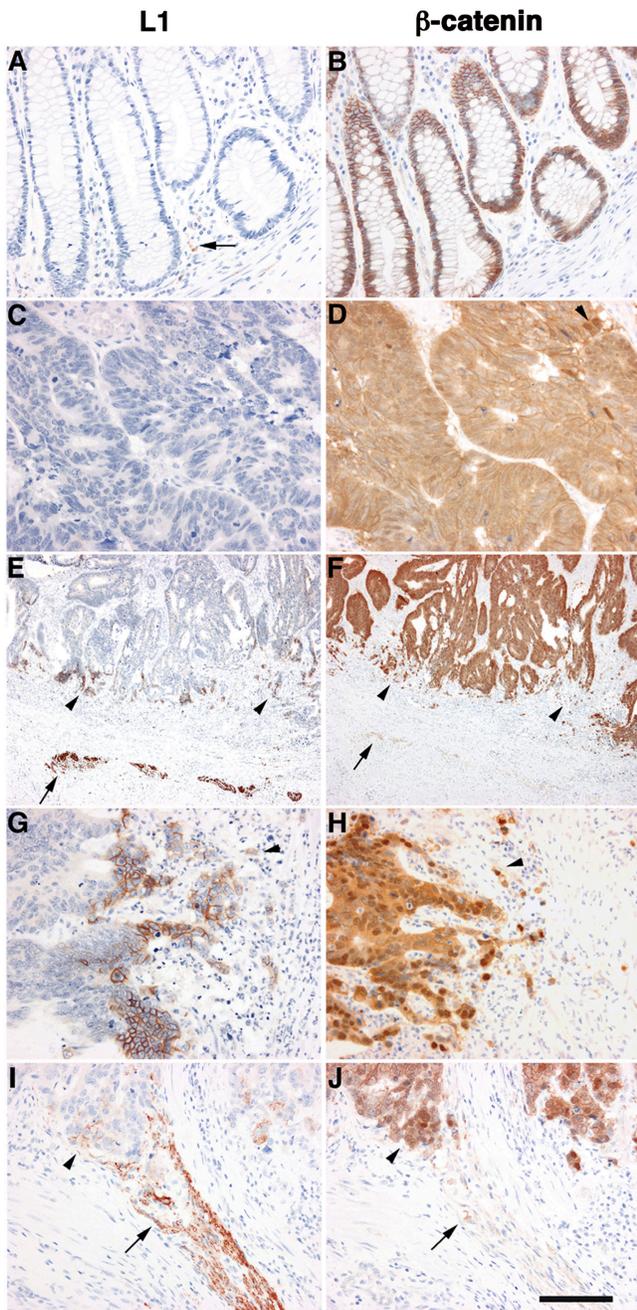


Figure 6. L1 is expressed in tumor cells at the invasive front of colorectal carcinomas often in contact with nerve axons. Immunohistochemical staining (brown) of L1 and β -catenin in adjacent serial sections with nuclear counterstaining (blue). (A and B) No L1 expression was seen in normal epithelial cells of the mucosa displaying membranous β -catenin. Weak staining for L1 was evident in intramucosal nerve axons (A, arrow). (C and D) No L1 expression was observed in the central, differentiated areas of the tumor. The arrowhead in D points to two cells displaying nuclear β -catenin. (E and F) Overview (at low magnification) of the invasive tumor front showing L1 in budding tumor cells, at the invasive front (arrowheads), and in nerve axons (arrows). (G and H) Higher magnification of E and F showing strong L1 expression in invading tumor cells and disseminated, single tumor cells expressing nuclear β -catenin (arrowheads). (I and J) Nuclear β -catenin positive tumor cells expressing L1 at the invasive front (arrowheads) grow in contact with L1 expressing nerve axons (arrows). Bar: (E and F) 375 μ m; (A–D and G–J) 75 μ m.

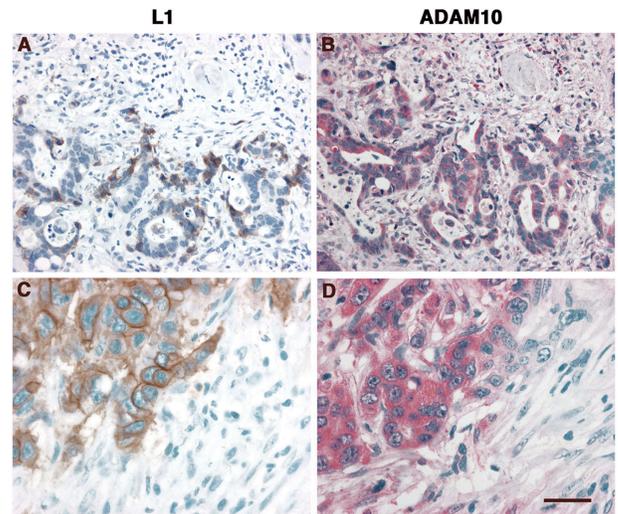


Figure 7. Coexpression of L1 and ADAM10 in tumor cells at the invasive front of colorectal carcinomas. Immunohistochemical stainings of L1 (brown) and ADAM10 (red) in adjacent serial sections, with nuclear counterstaining in blue. Bar: (A and B) 75 μ m; (C and D) 37.5 μ m.

paraffin sections of both normal and tumor tissue derived from surgical resections. In normal colon epithelium, we observed β -catenin staining at cell–cell contact sites (Fig. 6 B), and only sporadic staining for L1 along intramucosal nerve axons (Fig. 6 A, arrow). In the central, differentiated area of the tumor, β -catenin overexpression was detected in both the membrane and cytoplasm (Fig. 6 D), and often in the nuclei of cells (Fig. 6 D, arrowhead; unpublished data). Yet, we did not detect L1 in this part of the tumor tissue (Fig. 6 C) after analyzing 25 colorectal adenocarcinomas. In contrast, the invasive front of tumors displayed strong L1 expression in budding tumor cells at the interphase between tumor and normal tissue (Fig. 6, E and G, arrowheads) and in nerve axons (Fig. 6 E, arrow). This phenotype was apparent in 68% of the tumors. Higher magnification of the invasive tumor front displayed strong L1 expression in invading tumor cells and also in disseminated, single tumor cells (Fig. 6 G) expressing nuclear β -catenin (Fig. 6 H, arrowhead). Interestingly, in some sections where the nerve axons were more readily detectable, we observed L1 expressing cancer cells (Fig. 6 I, arrowhead) displaying nuclear β -catenin (Fig. 6 J, arrowhead) at the invasive front of the tumor, growing directly along L1 expressing nerve axons (Fig. 6 I, arrow). Because both these cancer and nerve cells express L1 on their surface, L1-mediated adhesive interactions among such cells might guide the further dissemination of invading tumor cells.

Because we found that L1 and ADAM10 expression in colon cancer cells is coregulated by changes in cell culture density (Fig. 1, D–F), and ADAM10 is involved in the cleavage and shedding of the extracellular domain of L1 (Gutwein et al., 2003), we determined the expression of ADAM10 in human colorectal cancer tissue. Staining with antibody to ADAM10 (and in adjacent sections for L1) of colon cancer tissue revealed that in 74% of tumors (19 tumors were analyzed), L1 and ADAM10 colocalized at the invasive front of the tumor tis-

sue (Fig. 7, A–D). These results indicate that L1 might be an important target gene of β -catenin-TCF signaling playing a key role, together with ADAM10, in the invasion of human colorectal cancer cells.

Discussion

L1 is a novel target of β -catenin-TCF signaling in colon cancer cells

In the present study, we demonstrated that in colon cancer cells the β -catenin-TCF transcriptional complex activates the *L1* gene, coding for a cell adhesion molecule mostly expressed in nerve cells. L1 levels were high in sparse cultures of colon cancer cells displaying characteristics of colorectal cancer cells at the invasive front of tumors (Brabletz et al., 2001). These include nuclear localization and signaling by β -catenin and down-regulation of E-cadherin by Slug, a negative transcriptional regulator of *E-cadherin* induced by β -catenin-TCF signaling (Conacci-Sorrell et al., 2003). Inhibition of β -catenin-TCF signaling in cultured colon cancer cells by inducible Δ NTCF, or suppression of β -catenin levels by inducible β -catenin siRNA, both reduced the expression of endogenous L1 in these cells (Fig. 2 F). In addition, the β -catenin-TCF complex in 293 cells and colorectal cancer cell lines activated the *L1* promoter reporter. Together, these results support the view that *L1* is a novel target gene of β -catenin signaling. This conclusion is in agreement with a previous study using DNA microarrays to determine genes down-regulated when wt APC was reintroduced into SW480 cells, and detected *L1* among such genes (Lin et al., 2001).

The biological activities of L1 in tumor cells

During tumor cell invasion, cancer cells often activate genes that normally promote the growth and migration of normal cells (in the course of various biological processes), to promote tumor cell metastasis. L1 is known for its important function in nerve cell axonal guidance, outgrowth, and pathfinding (Kamiguchi et al., 1998; Kenwrick et al., 2000), and its disruption causes serious damage to the nervous system, including mental retardation (De Angelis et al., 2002) and intestinal aganglionosis (Parisi et al., 2002). In addition, L1 was also found in other specialized sites including a subclass of lymphocytes, intestinal crypt cells, and kidney tubule epithelia (Kowitz et al., 1993; Thor et al., 1987; Debiec et al., 1998). We have shown that expression of L1 in NIH3T3 cells (that lack L1) confers several properties characteristic of cancer cells, including enhanced motility, cell transformation (manifested by the formation of foci in cells grown on plastic), and the ability to grow in low serum. Because NIH3T3 cells are preneoplastic, it remains to be determined whether L1 can transform normal, primary epithelial cells. The ability of L1 to confer NIH3T3 growth in the absence of serum is related to constitutively activated ERK displayed by these cells (Fig. 4 D). Activation of the MAPK pathway by L1 was reported previously (Schaefer et al., 1999), and recently linked to the enhanced motility of 3T3 fibroblasts and melanoma cells (Silletti et al., 2004). Because constitutive

ERK activation is considered a key step in cell transformation and the development of various tumors (Cowley et al., 1994; Hoshino et al., 1999; Welsh et al., 2000), its activation by L1 supports the notion that ERK-mediated gene expression is the major survival pathway allowing the proliferation of these cells in low serum. L1 expression also endowed these cells with increased tumorigenic capacity in nude mice, a finding similar to that reported with Nr-CAM, another L1 family member that is also a target gene of β -catenin-TCF signaling, overexpressed in melanoma and colon carcinoma tissue (Conacci-Sorrell et al., 2002b). The role of L1 in human colon cancer cell invasion and tumorigenesis is further supported by our data showing that its expression in human colon cancer cells lacking L1 increases haptotactic and invasive motility, and leads to faster tumor growth, whereas siRNA-mediated suppression of endogenous L1 in human colon cancer cells decreases cell motility (Fig. 5). Hence, L1 family members could function as potential promoters of colon cancer cell growth and invasion by being target genes of hyperactive β -catenin signaling.

Involvement of L1 and ADAM10 in human cancer invasion

Using the cell culture density model of human colon cancer cells, we found that the expression of L1 and of ADAM10 that mediates L1 cleavage and shedding (Mechtersheimer et al., 2001; Gutwein et al., 2003), is coregulated. Sparse cell cultures had much higher levels of L1 and ADAM10 protein and RNA than dense cultures (Fig. 1). Therefore, we analyzed their expression in human colorectal cancer tissue and compared it to that of β -catenin. We observed a very unique localization of L1 in a subpopulation of cancer cells expressing nuclear β -catenin at the invasive tumor front (in \sim 70% of the tumors), but not in the central, more differentiated part of the tumor (Fig. 6, C–H). This finding strongly supports a role for L1 in the invasive process of human colorectal cancer cells in vivo. ADAM10 expression was similarly enriched in the invasive front of such tumors (Fig. 7), indicating that L1 shedding into the surrounding microenvironment may play a role in dissemination of colon carcinoma cells. Interestingly, we often observed in very close proximity to the invasive front of tumors nerve axons, the only other cell type expressing L1 in colon mucosa (Fig. 6, E and J). The close apposition of invading tumor cells expressing L1, either on their surface (or as shedded molecules in their microenvironment), to L1 expressing nerve axons, could promote adhesive interactions between tumor and nerve cells, thereby contributing to colon cancer cell invasion using axons as tracks for migration. Studies using cocultures of neuronal and colon cancer cells should shed light on the possible mechanisms involving such interactions and the cross signaling between the different cell types.

β -Catenin-TCF activation of target genes is also known to regulate normal epithelial colon cell proliferation along the crypt-villus interface (van de Wetering et al., 2002). The Wnt/ β -catenin target genes involved, include the ephrins and their receptors (Battle et al., 2002). These molecules help establishing the boundary between proliferating and differentiated cells in the highly dynamic and renewing colon epithelium (Peifer,

2002). These same ephrins and their receptors are known to set segmental boundaries in the brain and determine axonal guidance in the retina. This points to another set of "brain-specific" cell adhesion molecules as key regulators of both normal and perhaps pathological behavior in the human colon.

L1 was previously detected in some normal tissues including leukocytes, intestinal crypt cells and kidney tubule epithelia (for review see Kenwrick et al., 2000), and the involvement of L1 in the motile and invasive properties of a variety of cultured cancer cell lines (lymphoma, lung carcinoma, melanoma) was also suggested (Kowitz et al., 1993; Miyahara et al., 2001; Thies et al., 2002). The shedding of the L1 ectodomain by ADAM10 and its activity in promoting cell adhesion by mechanisms involving integrin receptors was demonstrated in several cancer cell lines (Mechtersheimer et al., 2001). A recent study using transcriptome-scale selection to identify suppressors of breast carcinoma cell growth discovered L1 as a major positive regulator of carcinoma (but not normal) breast epithelial cell growth, and also of some colon and cervical cancer cell lines (Primiano et al., 2003). In agreement with these findings, we found that the proliferation of both SW480 and HCT116 cells is inhibited when they are incubated with anti-L1 antibody (Fig. 4), suggesting a key role for L1 in tumor cell growth. L1 and ADAM10 expression were also recently detected in ovarian and uterine carcinomas, and L1 shedding into the serum and ascites of such patients was suggested to be a predictive marker for their clinical outcome of (Fogel et al., 2003). Because endometrial carcinoma is often associated with aberrant activation of the β -catenin signaling pathway (Palacios and Gamallo, 1998; Moreno-Bueno et al., 2002), it could be that in endometrial tumors, as well, activation of *L1* is mediated by hyperactive β -catenin signaling. Together with the present study, these results imply that induction of L1 expression by β -catenin in malignant tumors might represent another case of opportunistic activation of target genes normally expressed in other tissues in the course of various biological processes. This property of cancer cells may facilitate their invasion and metastasis.

Materials and methods

Cell lines and cell culture

293, NIH3T3, SW480, LS174T, SW707, and HCT116 cells were maintained in DME with 10% bovine calf serum (BCS). NIH3T3 cells expressing puro^r constructs were cultured in 10 μ g/ml puromycin. HCT116 clones expressing the Tet-repressor were obtained from M. van de Wetering and H. Clevers (Hubrecht Laboratory, Utrecht, Netherlands), and were grown in RPMI with 5% FCS and Zeocin (500 μ g/ml). HCT116 cells expressing inducible, Δ NTCF4, or siRNA to β -catenin, were prepared as described previously (van de Wetering et al., 2003), and obtained from M. van de Wetering. They were cultured with Zeocin (500 μ g/ml) and Blasticidin (10 μ g/ml). Transgenes were induced with 1 μ g/ml doxycycline. Activation of MAPK was tested in cells starved for 18 h in DME. The cells were collected before, and at different times after incubation with 10% BCS.

Transfections and retroviral infection

Transient transfection of 293T cells was performed using calcium phosphate. SW480 and HCT116 cells were transfected using Lipofectamine 2000 (Invitrogen). In transactivation assays, 0.25 μ g of β -galactosidase plasmid was cotransfected with 1 μ g reporter plasmid and 2 μ g of either β -catenin, or cadherin tail constructs. Cells were plated in duplicates, lysed after 48 h, and luciferase and β -galactosidase levels were deter-

mined by enzyme assay kits purchased from Promega. Luciferase activity was normalized to β -galactosidase activity as internal transfection control. Fold induction of the *L1* and *Cyclin D1* promoters was calculated using empty reporter plasmid (PGL2 and PA3Luc, respectively). Retroviral infections were performed with pBabe-puro^r and pBabe-*L1* into 293T cells (2×10^6 cells/10 cm dish), by the calcium phosphate coprecipitation method, together with the ecotropic packaging vector pSV- ψ -E-MLV providing the helper function as described previously (Conacci-Sorrell et al., 2002b). Transfection of the *L1* siRNA sequence, 5'-GGAUGGUGUCCACUU-CAA-3' and 5'-UUUGAAGUGGACACCAUCC-3' and of control oligonucleotides obtained from QIAGEN was performed with annealed siRNAs using Oligofectamine (Life Technologies) and the transfected cells were analyzed after 72 h. LS174T cells stably expressing human L1 were established by transfection using Lipofectamine 2000, followed by neo^r selection. SW707 cells stably expressing human L1 were obtained by transfection with Superfect (Stratagene) and neomycin selection, followed by enrichment for L1 expression with mAb L1-11A and magnetic beads (Miltenyi Biotec).

Plasmids

The *L1* promoter reporter plasmid containing the transcription initiation site and 2.9-kb upstream sequences (Kallunki et al., 1997; Meech et al., 1999), including the four putative LEF/TCF-binding sites, was cloned into PGL2 (provided by F. Jones, Lois Pope LIFE Center, Miami, FL). Full-length *L1* cDNA (U52112) provided by V. Lemmon (University of Miami, Coral Gables, FL; Hlavin and Lemmon, 1991) was subcloned into the EcoRI site of pBabe-puro^r. Δ NTCF4 was provided by M. van de Wetering and H. Clevers, as were the TOPFLASH and FOPFLASH synthetic promoter reporter plasmids. Δ LEF was a gift from R. Kemler (Max Planck Institute for Immunobiology, Freiburg, Germany). The mutant β -catenin S33Y, *Cyclin D1* reporter plasmid, dominant positive LEF-1 construct (Shtutman et al., 1999) and the plasmid coding for the cytoplasmic domain of E-cadherin (Cad tail; Sadot et al., 1998) were described previously.

RT-PCR and electrophoretic mobility shift assays

RT-PCR for *L1* was performed using the primers: ACGGGCAACAACAGCAAC and CGGCTTCCTGTCAATCATG, and for GAPDH: ACCACAGTCCATGCCATCAC and TCCACCACCCTGTGCTGTA. The primers for E-cadherin and ADAM10 and PCR conditions were as described previously (Fogel et al., 2003). For electrophoretic mobility shift assays, double-stranded DNA oligonucleotides containing the putative LEF/TCF-binding sites of the *L1* promoter (Fig. 2 C) and 10 adjacent nucleotides upstream and 10 downstream were used. SW480 cells were harvested, incubated for 15 min in low-salt buffer, NP-40 was added, nuclei were pelleted by centrifugation, and nuclear proteins extracted with high-salt buffer as described previously (Shtutman et al., 1999). For DNA-binding assays, 6 μ g of nuclear extract were used. Increasing volumes of polyclonal β -catenin antibody were added to the binding reaction to analyze DNA mobility supershift.

Immunofluorescence

Cells cultured on glass coverslips were permeabilized with 0.5% Triton X-100 and fixed with 3% PFA in PBS. The coverslips were incubated with polyclonal antibody against the extracellular domain of L1 provided by V. Lemmon (Schaefer et al., 1999), or the mAb L1-11A (a subclone derived from hybridoma UJ127.11; Mechtersheimer et al., 2001). The mAb against β -catenin was purchased from Transduction Laboratories. The secondary antibodies were Alexa 488-conjugated goat anti-mouse or anti-rabbit IgG (Molecular Probes) and Cy3-labeled goat anti-mouse, or anti-rabbit IgG (Jackson ImmunoResearch Laboratories), as described by Simcha et al. (1998). Images were acquired using the DeltaVision system (Applied Precision) equipped with a microscope (model Axiovert 100; Carl Zeiss Microimaging, Inc.) and Photometrics 300 series scientific-grade cooled CCD camera, reading 12-bit images, and using the 63 \times /1.4 NA plan-Neofluar objective. Adjustments of brightness, contrast, color balance, and final size of images was processed using Adobe Photoshop 5.5.

Western blotting, cell growth rate and wound healing

Additional antibodies used in Western blotting were mouse anti-tubulin (Sigma-Aldrich), mouse anti-E-cadherin (Transduction Laboratories), goat anti-ADAM10 (R&D Systems), mouse anti-MAPK and rabbit antiphosphorylated MAPK (Sigma-Aldrich). The Western blots were developed using the ECL method (Amersham Biosciences). For cell growth rate, 5×10^3 cells were plated into 24-well dishes and their number determined every 24 h for 6 d in triplicates. Cells were also grown in the presence of anti-

body against L1-CAM, at 1:200 dilution with HCT116 and 1:50 with SW480 cells. Anti-L1 antibody (boiled for 10 min) was used as control. A "wound" was introduced into a confluent monolayer of cells with the tip of a micropipette as described previously (Conacci-Sorrell et al., 2002b), the culture medium replaced with fresh medium, and 40 μ g/ml of Mitomycin C was added to inhibit cell proliferation. After 24 h, the cells were fixed with ice-cold methanol, stained with Giemsa, and photographed.

FACS analysis, transmigration, and matrigel invasion assays

For FACS analysis, the cells were stained with mAb to L1 and PE-conjugated secondary antibodies, or with secondary antibody alone as control (Mechtersheimer et al., 2001). Stained cells were analyzed with a FAC-Scan using the Cellquest software (Becton Dickinson). Haptotactic cell migration assays (toward 10 μ g/ml fibronectin coated on the backside of the filter) was performed in Transwell chambers (Costar; Mechtersheimer et al., 2001). After 16 h, cells that migrated to the backside of the filter were stained with crystal violet solution and the OD of the eluted dye was determined at 595 nm in an ELISA reader. Invasion assays were performed with 24-well BioCoat Matrigel Invasion Chambers (Becton Dickinson) using 5×10^4 cells in serum-free DME and plated onto either control or matrigel-coated filters. Conditioned medium from 3T3 cells was placed in the lower chambers as chemoattractant. After 22 h in culture, cells were removed from the upper surface of the filter by scraping with a cotton swab. Cells that invaded through the matrigel and were adherent to the bottom of the membrane were stained with crystal violet solution. The cell-associated dye was eluted with 10% acetic acid and its OD at 595 nm determined. Each experiment was done in quadruplicates and the mean values \pm SEM are presented.

Tumorigenicity assays

NIH3T3 cells (10^5 and 2.5×10^5) expressing retrovirally transduced L1, or the empty (puro') vector, or LS174T and LS174T-L1 cells (10^6 cells), were injected subcutaneously into 6-wk-old CD1 nude male mice. Control neo'-LS174T cells were injected on one side, and LS174T-L1 on the other side of each animal in the presence or absence of matrigel (250 μ l/animal), generously provided by H. Kleinman (National Institutes of Health, Bethesda, MD). Groups of six mice were followed for 2 wk, when the size of tumors reached \sim 1–2 cm in diameter. After sacrificing the mice, the tumors were excised and weighed. Normal subcutaneous tissue was also excised, and RNA was prepared from normal and tumor tissue for RT-PCR. SW707 and SW707-L1 cells (10^7 cells/animal) were injected into the flanks of 4 scid/nod mice in each group, and tumor volume was determined at different times after injection by measuring tumor diameter.

Tissue immunohistochemistry

Immunohistochemistry was performed as previously described (Brabletz et al., 1999). In brief, 3- μ m serial sections from 25 formalin-fixed, paraffin-embedded, colorectal adenocarcinomas were deparaffinized, rehydrated, and pretreated for antigen retrieval by microwave treatment for 20 min in 10 μ M of citrate buffer, pH 6.0. To detect L1 and ADAM10, the antibodies described above were used. Biotinylated rabbit anti-mouse Ig anti-serum, or rabbit anti-goat antiserum (both diluted 1:200; DakoCytomation), served as secondary antibody. To detect L1 and β -catenin (brown staining), the Envision system was used for ADAM10 (red staining), and Strept/AB (for β -catenin) was used according to the manufacturer's protocol (DakoCytomation). After rinsing with water, sections were counterstained with hemalaun (Merck), dehydrated, and covered with coverslips.

We are grateful to our colleagues, Vance Lemmon, Marc van de Wetering, Hans Clevers, Frederick Jones, Rolf Kemler, and Hynda Kleinman for providing reagents.

These studies were supported by grants to A. Ben-Ze'ev from the German-Israeli Foundation for Scientific Research and Development (GIF), the MD Moross Institute for Cancer Research, Israel Cancer Research Fund, a grant from the Estate of Bronia Hacker, La Fondation Raphael et Regina Levy and from the Yad Abraham Center for Cancer Research and Diagnosis. P. Altevogt and T. Brabletz were supported by grants from the Deutsche Krebshilfe (Schwerpunktprogramm: Invasion and Migration) and Deutsche Forschungsgemeinschaft.

Submitted: 9 August 2004

Accepted: 5 January 2005

References

Barker, N., and H. Clevers. 2001. Tumor environment: a potent driving force in colorectal cancer? *Trends Mol. Med.* 7:535–537.

- Battle, E., J.T. Henderson, H. Beghtel, M.M. van den Born, E. Sancho, G. Huls, J. Meeldijk, J. Robertson, M. van de Wetering, T. Pawson, and H. Clevers. 2002. β -Catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. *Cell*. 111:251–263.
- Ben-Ze'ev, A., and B. Geiger. 1998. Differential molecular interactions of β -catenin and plakoglobin in adhesion, signaling and cancer. *Curr. Opin. Cell Biol.* 10:629–639.
- Bienz, M., and H. Clevers. 2000. Linking colorectal cancer to Wnt signaling. *Cell*. 103:311–320.
- Brabletz, T., A. Jung, S. Dag, F. Hlubek, and T. Kirchner. 1999. β -Catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer. *Am. J. Pathol.* 155:1033–1038.
- Brabletz, T., A. Jung, S. Reu, M. Porzner, F. Hlubek, L.A. Kunz-Schughart, R. Knuechel, and T. Kirchner. 2001. Variable β -catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. *Proc. Natl. Acad. Sci. USA.* 98:10356–10361.
- Conacci-Sorrell, M., J. Zhurinsky, and A. Ben-Ze'ev. 2002a. The cadherin-catenin adhesion system in signaling and cancer. *J. Clin. Invest.* 109:987–991.
- Conacci-Sorrell, M.E., T. Ben-Yedidia, M. Shtutman, E. Feinstein, P. Einat, and A. Ben-Ze'ev. 2002b. Nr-CAM is a target gene of the β -catenin/LEF-1 pathway in melanoma and colon cancer and its expression enhances motility and confers tumorigenesis. *Genes Dev.* 16:2058–2072.
- Conacci-Sorrell, M., I. Simcha, T. Ben-Yedidia, J. Blechman, and A. Ben-Ze'ev. 2003. Autoregulation of E-cadherin expression by cadherin-cadherin interactions: the roles of β -catenin signaling, Slug and MAPK. *J. Cell Biol.* 163:847–857.
- Cowley, S., H. Paterson, P. Kemp, and C. Marshall. 1994. Activation of MAP kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. *Cell*. 77:841–852.
- Crawford, H., B. Fingleton, L. Rudolph-Owen, K. Goss, B. Rubinfeld, P. Polakis, and L. Matrisian. 1999. The metalloproteinase matrilysin is a target of β -catenin transactivation in intestinal tumors. *Oncogene*. 18:2883–2891.
- De Angelis, E., A. Watkins, M. Schafer, T. Brummendorf, and S. Kenwick. 2002. Disease-associated mutations in L1 CAM interfere with ligand interactions and cell-surface expression. *Hum. Mol. Genet.* 11:1–12.
- Debiec, H., E. Christensen, and P.M. Ronco. 1998. The cell adhesion molecule L1 is developmentally regulated in the renal epithelium and is involved in kidney branching morphogenesis. *J. Cell Biol.* 143:2067–2079.
- Fogel, M., P. Gutwein, S. Mechtersheimer, S. Riedle, A. Stoeck, A. Smirnov, L. Edler, A. Ben-Arie, M. Huszar, and P. Altevogt. 2003. L1 expression as a predictor of progression and survival in patients with uterine and ovarian carcinomas. *Lancet*. 362:869–875.
- Grabl, D., M. Kuhl, and D. Wedlich. 1999. The Wnt/Wg signal transducer β -catenin controls fibronectin expression. *Mol. Cell Biol.* 19:5576–5587.
- Gutwein, P., S. Mechtersheimer, S. Riedle, A. Stoeck, D. Gast, S. Joumaa, H. Zentgraf, M. Fogel, and D.P. Altevogt. 2003. ADAM10-mediated cleavage of L1 adhesion molecule at the cell surface and in released membrane vesicles. *FASEB J.* 17:292–295.
- He, T., A. Sparks, C. Rago, H. Hermeking, L. Zawel, L. da Costa, P. Morin, B. Vogelstein, and K. Kinzler. 1998. Identification of c-MYC as a target of the APC pathway. *Science*. 281:1509–1512.
- Hlavin, M.L., and V. Lemmon. 1991. Molecular structure and functional testing of human L1CAM: an interspecies comparison. *Genomics*. 11:416–423.
- Hlubek, F., A. Jung, N. Kotzor, T. Kirchner, and T. Brabletz. 2001. Expression of the invasion factor laminin γ 2 in colorectal carcinomas is regulated by β -catenin. *Cancer Res.* 61:8089–8093.
- Hlubek, F., S. Spaderna, A. Jung, T. Kirchner, and T. Brabletz. 2004. β -Catenin activates a coordinated expression of the proinvasive factors laminin-5 γ 2 chain and MT1-MMP in colorectal carcinomas. *Int. J. Cancer*. 108:321–326.
- Hoshino, R., Y. Chatani, T. Yamori, T. Tsuruo, H. Oka, O. Yoshida, Y. Shimada, S. Ari-i, H. Wada, J. Fujimoto, and M. Kohno. 1999. Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors. *Oncogene*. 18:813–822.
- Kallunki, P., G.M. Edelman, and F.S. Jones. 1997. Tissue-specific expression of the L1 cell adhesion molecule is modulated by the neural restrictive silencer element. *J. Cell Biol.* 138:1343–1354.
- Kamiguchi, H., M.L. Hlavin, and V. Lemmon. 1998. Role of L1 in neural development: what the knockouts tell us. *Mol. Cell Neurosci.* 12:48–55.
- Kenwick, S., A. Watkins, and E. De Angelis. 2000. Neural cell recognition molecule L1: relating biological complexity to human disease mutations. *Hum. Mol. Genet.* 9:879–886.
- Kowitz, A., G. Kadmon, H. Verschuere, L. Remels, P. De Baetselier, M. Hubbe, M. Schachner, V. Schirmacher, and P. Altevogt. 1993. Expression of L1 cell adhesion molecule is associated with lymphoma growth and metastasis. *Clin. Exp. Metastasis*. 11:419–429.

- Lin, Y.M., K. Ono, S. Satoh, H. Ishiguro, M. Fujita, N. Miwa, T. Tanaka, T. Tsunoda, K.C. Yang, Y. Nakamura, and Y. Furukawa. 2001. Identification of AF17 as a downstream gene of the β -catenin/T-cell factor pathway and its involvement in colorectal carcinogenesis. *Cancer Res.* 61:6345–6349.
- Mann, B., M. Gelos, A. Siedow, M. Hanski, A. Gratchev, M. Ilyas, W. Bodmer, M. Moyer, E. Riecken, and H. Buhr. 1999. Target genes of β -catenin-T cell-factor/lymphoid-enhancer-factor signaling in human colorectal carcinomas. *Proc. Natl. Acad. Sci. USA.* 96:1603–1608.
- Meckersheimer, S., P. Gutwein, N. Agmon-Levin, A. Stoeck, M. Oleszewski, S. Riedle, M. Fogel, V. Lemmon, and P. Altevogt. 2001. Ectodomain shedding of L1 adhesion molecule promotes cell migration by autocrine binding to integrins. *J. Cell Biol.* 155:661–673.
- Meech, R., P. Kallunki, G.M. Edelman, and F.S. Jones. 1999. A binding site for homeodomain and Pax proteins is necessary for L1 cell adhesion molecule gene expression by Pax-6 and bone morphogenetic proteins. *Proc. Natl. Acad. Sci. USA.* 96:2420–2425.
- Miyahara, R., F. Tanaka, T. Nakagawa, K. Matsuoka, K. Isii, and H. Wada. 2001. Expression of neural cell adhesion molecules (polysialylated form of neural cell adhesion molecule and L1-cell adhesion molecule) on resected small cell lung cancer specimens: in relation to proliferation state. *J. Surg. Oncol.* 77:49–54.
- Moreno-Bueno, G., D. Hardisson, C. Sanchez, D. Sarrio, R. Cassia, G. Garcia-Rostan, J. Prat, M. Guo, J.G. Herman, X. Matias-Guiu, et al. 2002. Abnormalities of the APC/ β -catenin pathway in endometrial cancer. *Oncogene.* 21:7981–7990.
- Morin, P., A. Sparks, V. Korinek, N. Barker, H. Clevers, B. Vogelstein, and K. Kinzler. 1997. Activation of β -catenin-Tcf signaling in colon cancer by mutations in β -catenin or APC. *Science.* 275:1787–1790.
- Palacios, J., and C. Gamallo. 1998. Mutations in the β -catenin gene (CTNNB1) in endometrioid ovarian carcinomas. *Cancer Res.* 58:1344–1347.
- Parisi, M.A., R.P. Kapur, I. Neilson, R.M. Hofstra, L.W. Holloway, R.C. Michaelis, and K.A. Leppig. 2002. Hydrocephalus and intestinal aganglionosis: is LICAM a modifier gene in Hirschsprung disease? *Am. J. Med. Genet.* 108:51–56.
- Peifer, M. 2002. Developmental biology: colon construction. *Nature.* 420:274–275.
- Polakis, P. 2000. Wnt signaling and cancer. *Genes Dev.* 14:1837–1851.
- Primiano, T., M. Baig, A. Maliyekkel, B.D. Chang, S. Fellars, J. Sadhu, S.A. Axenovich, T.A. Holzmayr, and I.B. Roninson. 2003. Identification of potential anticancer drug targets through the selection of growth-inhibitory genetic suppressor elements. *Cancer Cell.* 4:41–53.
- Sadot, E., I. Simcha, M. Shtutman, A. Ben-Ze'ev, and B. Geiger. 1998. Inhibition of β -catenin-mediated transactivation by cadherin derivatives. *Proc. Natl. Acad. Sci. USA.* 95:15339–15344.
- Schaefer, A.W., H. Kamiguchi, E.V. Wong, C.M. Beach, G. Landreth, and V. Lemmon. 1999. Activation of the MAPK signal cascade by the neural cell adhesion molecule L1 requires L1 internalization. *J. Biol. Chem.* 274:37965–37973.
- Shtutman, M., J. Zhurinsky, I. Simcha, C. Albanese, M. D'Amico, R. Pestell, and A. Ben-Ze'ev. 1999. The cyclin D1 gene is a target of the β -catenin/LEF-1 pathway. *Proc. Natl. Acad. Sci. USA.* 96:5522–5527.
- Silletti, S., M. Yebra, B. Perez, V. Cirulli, M. McMahon, and A.M. Montgomery. 2004. Extracellular signal-regulated kinase (ERK)-dependent gene expression contributes to L1 cell adhesion molecule-dependent motility and invasion. *J. Biol. Chem.* 279:28880–28888.
- Simcha, I., M. Shtutman, D. Salomon, J. Zhurinsky, E. Sadot, B. Geiger, and A. Ben-Ze'ev. 1998. Differential nuclear translocation and transactivation potential of β -catenin and plakoglobin. *J. Cell Biol.* 141:1433–1448.
- Takahashi, M., T. Tsunoda, M. Seiki, Y. Nakamura, and Y. Furukawa. 2002. Identification of membrane-type matrix metalloproteinase-1 as a target of the β -catenin/Tcf4 complex in human colorectal cancers. *Oncogene.* 21:5861–5867.
- Tetsu, O., and F. McCormick. 1999. β -Catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature.* 398:422–426.
- Thies, A., M. Schachner, I. Moll, J. Berger, H.J. Schulze, G. Brunner, and U. Schumacher. 2002. Overexpression of the cell adhesion molecule L1 is associated with metastasis in cutaneous malignant melanoma. *Eur. J. Cancer.* 38:1708–1716.
- Thor, G., R. Probstmeier, and M. Schachner. 1987. Characterization of the cell adhesion molecules L1, N-CAM and J1 in the mouse intestine. *EMBO J.* 6:2581–2586.
- van de Wetering, M., I. Oving, V. Muncan, M.T. Pon Fong, H. Brantjes, D. van Leenen, F.C. Holstege, T.R. Brummelkamp, R. Agami, and H. Clevers. 2003. Specific inhibition of gene expression using a stably integrated, inducible small-interfering-RNA vector. *EMBO Rep.* 4:609–615.
- van de Wetering, M., E. Sancho, C. Verweij, W. de Lau, I. Oving, A. Hurlstone, K. van der Horn, E. Batlle, D. Coudreuse, A.P. Haramis, et al. 2002. The β -catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell.* 111:241–250.
- Welsh, D., T. Sakamaki, R. Pioquinto, T. Leonard, S. Goldberg, Q. Hon, R. Erikson, M. Rieber, M.S. Rieber, D. Hicks, et al. 2000. Transfection of constitutively active mitogen-activated protein/extracellular signal-regulated kinase confers tumorigenic and metastatic potentials to NIH3T3 cells. *Cancer Res.* 60:1552–1556.
- Wielenga, V.J., R. Smits, V. Korinek, L. Smit, M. Kielman, R. Fodde, H. Clevers, and S.T. Pals. 1999. Expression of CD44 in Apc and Tcf mutant mice implies regulation by the WNT pathway. *Am. J. Pathol.* 154:515–523.